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TITLE: Targeting Prostate Cancer Stemlike Cells through Cell Surface-Expressed GRP78

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

This proposal investigates cell surface GRP78 as a target for eliminating prostate cancer stem-like cells. In period 2, we demonstrated that prostate cancer cells are heterogeneous, being composed of cell surface GRP78(+) and cell surface GRP78(-) tumor cells. In the current period (period 3), we implement cell sorting to isolate cell surface GRP78(+) and cell surface GRP78(-) prostate cancer cell populations. We demonstrate that the cell surface GRP78(+) tumor cells, but not the cell surface GRP78(-) cells, exhibit self renewing activity in a sphere forming assay (an activity associated with cancer stemness). We also show that the cell surface GRP78-expressing subpopulation of cells supports nuclear Akt/GSK-3/Snail-1 signaling. These findings are important because they are the first to suggest that GRP78 regulates the activity of nuclear Akt, which has been implicated in therapy resistance (Bozulic, L, Surucu, B, Hynx, D, and Hemmings, BA. 2008. PKBalpha/Akt1 acts downstream of DNA-PK in the DNA double-strand break response and promotes survival. Mol. Cell. 30:203-13). Due to a loss of critical personnel in the laboratory of Dr. Chi (co-investigator), we were unable to test the hypothesis that cell surface GRP78(+) prostate cancer cells exhibit increased tumor initiating activity (compared to negative cells) using a xenograft model. We obtained a one year, no-cost extension for the remaining budget of the grant, which will allow us to perform these important animal studies, thus completing the original tasks outlined in the approved statement of work.

15. SUBJECT TERMS
prostate cancer, cell surface GRP78, cancer stem cell, Snail-1

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INTRODUCTION:

In our last progress report, we provided data indicating that prostate cancer cells are heterogeneous, being composed of cell surface GRP78(+) and GRP78(-) tumor cells. By cell sorting we showed that the GRP78(+) prostate cancer cells, but not the GRP78(-) cells, exhibited cancer stem-like cell behavior (i.e. demonstrated sphere forming ability in non-adherent conditions in the absence of serum). Furthermore, an antibody that binds to the carboxyl terminal domain of GRP78 inhibited sphere forming ability of GRP78(+) prostate cancer cells. In the current grant period, we studied whether signaling pathways associated with cell surface GRP78 (Akt/GSK-3/Snail-1) were upregulated in GRP78(+) relative to GRP78(-) prostate cancer cells. Our results in this period demonstrate that GRP78(+) cells, relative to GRP78(-) cells, express increased phospho-Akt , increased phospho-GSK-3, and increased Snail-1. These studies show that cell surface GRP78(+) prostate cancer stem-like cells support an Akt/GSK-3/Snail-1 signaling axis. In our previous studies demonstrating that cell surface GRP78 activates Akt in prostate cancer cells (*1*), we assumed that activated Akt was located in the cytosol. Surprisingly, by separating nuclear from cytosolic proteins, we observed in the current studies that the majority of phospho-Akt was localized in the nucleus of cell surface GRP78(+) tumor cells. Importantly, Akt could not be detected in the nucleus of GPR78(-) prostate cancer cells. Based on previous work showing that nuclear Akt can drive therapy resistance (*2*), we are currently exploring whether cell surface GRP78(+) supports the growth of multidrug resistant, prostate cancer stem-like cells by activating nuclear Akt. Follow-up studies will investigate the ability of our monoclonal antibody directed against the carboxyl terminus of GRP78 to inhibit: 1) nuclear Akt activity in cell surface GRP78(+) prostate cancer cells and 2) cancer stem behaviors, including multidrug resistance. We were unable to initiate animal studies testing tumor initiating activity of GRP78-sorted prostate cancer cells because Dr. Chi, our animal study co-investigator, lost his animal technician. Accordingly, we obtained a one-year, no cost extension, allowing Dr. Chi to perform these important animal studies upon hiring replacement personnel.
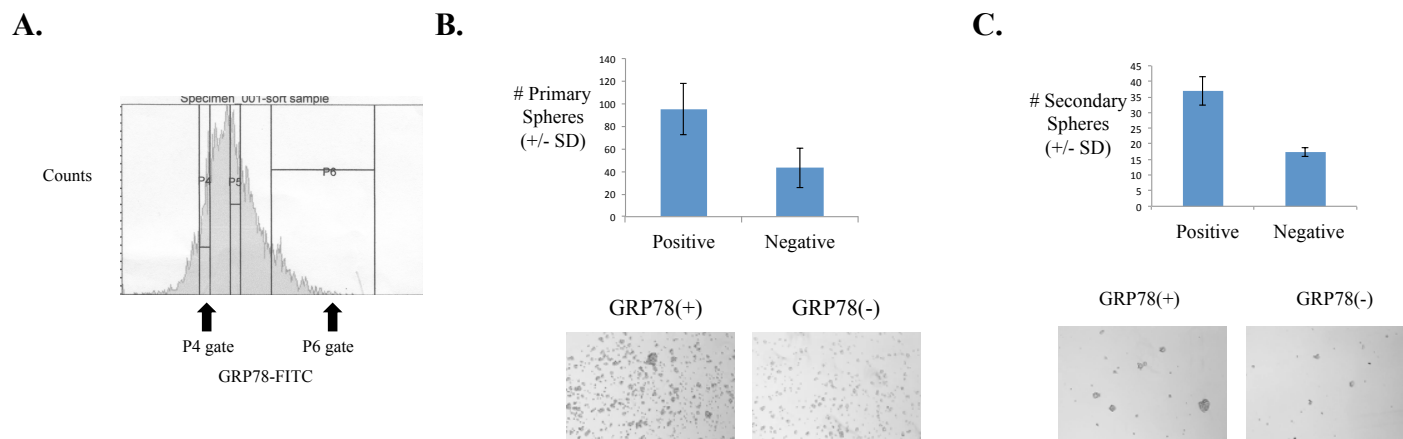
KEYWORDS: GRP78, prostate cancer stem-like cell, prostaspheres

OVERALL PROJECT SUMMARY (Tasks refer to those outlined in approved Statement of Work):

Task 6: Investigate the relative ability of cell surface GRP78(+) and cell surface GRP78(-) prostate cancer cells to grow as self-renewing prostaspheres in vitro.

In the last progress report, we reported a sorting strategy for isolating cell surface GRP78(+) and cell surface GRP78(-) prostate cancer cells (DU145) with the goal of testing the relative abilities of these two populations to grow as self-renewing spheres, a property of cancer stem-like cells. We showed that GRP78(+) tumor cells were more efficient in sphere formation than GRP78(-) tumor cells. In the current period, we sought to determine if cell surface GRP78(+) tumor cells exhibited self-renewing activity by harvesting primary spheres and reseeding these cells into a secondary sphere assay. Using our cell sorting strategy, cell surface GRP78(+) and cell surface GRP78(-) DU145 cell populations were isolated (Fig. 1A). As shown in Figure 1B and C, cell surface GRP78(+) tumor cells were more efficient at primary and secondary sphere formation than GRP78(-) tumor cells, indicating that the former exhibited self-renewing activity.
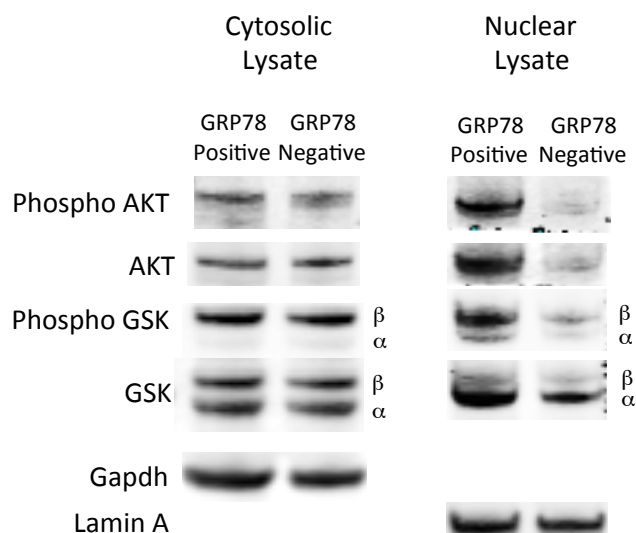
Figure 1: Measuring self-renewing activity of cell surface GRP78(+) and cell surface GRP78(-) DU145 prostate cancer cells. DU145 cells were harvested with 2 mM EDTA and stained with anti-GRP78-alexa fluor 488 (10 ug/10^6 cells) and 7AAD to exclude dead cells. **A.** Cells were sorted into GRP78-positive (P6 gate) and GRP78-negative (P4 gate) populations**. B.** Sorted populations were grown as prostaspheres. Prostasphere number from three wells (+/- SEM) was determined on d8 (top panel). Pictures of representative wells are shown (bottom panel). **C.** Primary prostaspheres were dissociated with trypsin and seeded at equal numbers into secondary sphere assays. Prostasphere numbers from three wells were determined on d13. Pictures of representative wells are shown (bottom panel).

A.

B.

C.

Task 3: Determine relative activities of Akt and its substrate GSK-3 in adherent prostate cancer cells and in prostate cancer stem-like cells.

Having shown by cell sorting that cell surface GRP78(+) but not cell surface GRP78(-) prostate cancer cells can grow as self-renewing spheres, we next sought to address the hypothesis that cell surface GRP78-stimulated Akt signaling drives this activity. Accordingly, we extracted nuclear and cytosolic proteins from sorted GRP78(+) and GRP78(-) prostate cancer cells, and performed immunoblotting to measure levels of phospho-Akt and Akt. Classical Akt signaling occurs in the cytosol. Thus we were surprised to observe that the predominant phospho-Akt signal in GRP78(+) tumor cells was localized in the nucleus. As demonstrated in Fig. 2, cell GRP78(+) cells exhibited a 100-fold increase in nuclear phospho-Akt levels compared to that in GRP78(-) cells (as determined by densitometry). In contrast, cytosolic phospho-Akt levels were equal in GRP78(+) and GRP78(-) cells. These studies are the first to show an association between cell surface GRP78 expression in prostate cancer cells and nuclear Akt signaling. We also performed immunoblotting for GSK-3, a direct substrate of Akt. As shown in Fig. 2, GRP78(+) prostate cancer cells exhibited a 2.4 fold increase in phospho-GSK-3 levels compared to that in GRP78(-) cells. Interestingly, phosphorylated GSK-3 protein in cell surface GRP78(+) tumor cells was localized in the nucleus. GSK-3 exists as two isoforms (alpha and beta). As shown in Fig. 3, cell surface GRP78 expression was associated with increased phospho-GSK-3 alpha, but not increased phospho-GSK-3 beta.
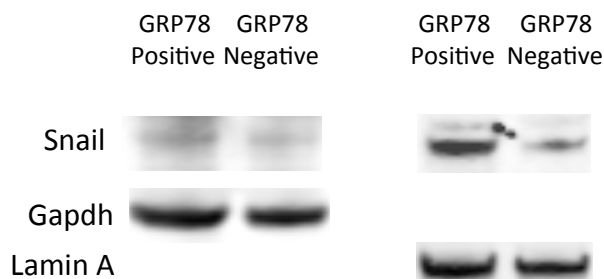
Figure 2 (next page): Cell surface GRP78 expression in prostate cancer cells is associated with activation of a nuclear Akt/GSK-3 signaling axis. Cell surface GRP78(+) and (-) DU145 prostate cancer cells were obtained by cell sorting, as in Fig. 1. Nuclear and cytosolic proteins were extracted using our previously published methods. Equivalent amounts of protein were subjected to SDS-PAGE, and immunoblotted with phospho-Akt, Akt, phospho-GSK-3, GSK-3, GAPDH, and Lamin A antibodies, followed by IRdye conjugated secondary antibody.

| | Cytosolic Lysate | | Nuclear Lysate | |
| | GRP78 Positive | GRP78 Negative | GRP78 Positive | GRP78 Negative |

Phospho AKT

AKT

Phospho GSK — β / α

GSK — β / α

Gapdh

Lamin A

Task 4: Investigate the relative expression of Snail-1, a GSK-3 target, in adherent prostate cancer cells and in prostate cancer stem-like cells.

We and others have previously shown that GSK-3 is an important regulator of the expression/nuclear localization of Snail-1 (*3, 4*), a transcription factor that drives cancer stem cell behaviors (*5*). Accordingly, we next investigated relative expression of nuclear and cytosolic Snail-1 in cell surface GRP78(+) and cell surface GRP78(-) DU145 cells obtained by cell sorting. GRP78(+) tumor cells exhibited a 2.5-fold increase in nuclear Snail-1 levels compared to GRP78(-) tumor cells, as determined by densitometry (Fig. 3).

> **Figure 3: Cell surface GRP78 expression in prostate cancer cells is associated with increased levels of nuclear Snail-1, a transcription factor associated with cancer stemness.** Cell surface GRP78(+) and (-) DU145 prostate cancer cells were obtained by cell sorting, as in Fig. 1. Nuclear and cytosolic proteins were extracted using our previously published methods. Equivalent amounts of protein were subjected to SDS-PAGE, and immunoblotted with Snail-1, GAPDH, and Lamin A antibodies, followed by IRdye conjugated secondary antibody. Protein bands were detected using a Licor Odyssey Fluorescent scanner.

| | GRP78 Positive | GRP78 Negative | GRP78 Positive | GRP78 Negative |

Snail

Gapdh

Lamin A

Task 8: Determine the relative ability of cell surface GRP78(+) and cell surface GRP78(-) prostate cancer cells to initate tumor growth in a xenograft model.

An important behavior of cancer stem-like cells is their enhanced tumor initiating activity relative to cancer cells lacking stem behaviors. Thus, we next sought to determine the relative abilities of sorted cell surface GRP78(+) and (-) prostate cancer cells to establish tumors in a xenograft model. These studies were to be performed in the laboratory of Dr. Ashley Chi. Unfortunately, Dr. Chi's animal research technician left the Chi lab in July of 2015. We thus obtained a one-year, no-cost extension to allow us to perform these important animal studies once Dr. Chi has hired a qualified animal technician.

KEY RESEARCH ACCOMPLISHMENTS:

- Showed that cell surface GRP78(+) prostate cancer cells are more efficient at prostasphere formation and self renewal than cell surface GRP78(-) prostate cancer cells.
- Demonstrated that cell surface GRP78 expression is associated with the activation of an Akt/GSK-3 signaling axis.
- Provided the first evidence that cell surface GRP78(+) prostate cancer cells support ***nuclear*** Akt signaling.
- Showed that Snail-1, a transcription factor associated with stemness and regulated by Akt/GSK-3 signaling, is increased in GRP78(+) tumor cells relative to GRP78(-) tumor cells.

CONCLUSION: By performing cell sorting on DU145 prostate cancer cells, we showed that cell surface GRP78(+) prostate cancer cells exhibit increased ability to grow as self-renewing prostaspheres [compared to cell surface GRP78(-) prostate cancer cells]. We also demonstrated that cell surface GRP78(+) tumor cells exhibit increased Akt activity, and that this activity is localized to the nucleus. Cell surface GRP78(+) tumor cells have increased phospho-GSK-3 levels, as well as increased levels of the nuclear transcription factor Snail-1, an established determinant of stemness.   These accomplishments in year 3 provide a foundation for determining the relative tumor initiating activity of GRP78+ and GRP78- prostate cancer cells in a xenograft model during our approved no cost extension period for this grant. We will also study efficacy of monoclonal antibodies directed against the carboxyl terminus of GRP78 to inhibit nuclear Akt/GSK-3/Snail-1 signaling and cancer stem cell behaviors in prostate cancer cells.

PUBLICATIONS, ABSTRACT, AND PRESENTATIONS:

Nothing to report for this period.

INVENTIONS, PATENTS, AND LICENSES: Nothing to report

REPORTABLE OUTCOMES:
1. Our results from this period are the first to show an association between cell surface GRP78 signaling and nuclear Akt activity in prostate cancer cells. Based on recently published findings that nuclear Akt drives therapy resistance, our findings provide an important foundation for future studies investigating a function for cell surface GRP78/nuclear Akt signaling in regulating prostate cancer stemness/multidrug resistance.

OTHER ACHIEVEMENTS: Nothing to report

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APPENDICES: N/A